AWARD NUMBER: W81XWH-13-1-0352

TITLE: Microenvironment-Programmed Metastatic Prostate Cancer Stem Cells (mPCSCs)

PRINCIPAL INVESTIGATOR: Dean G. Tang, M.D., Ph.D.

CONTRACTING ORGANIZATION: The University of Texas MD Anderson Cancer Center
Houston, TX 78957

REPORT DATE: October 2014

TYPE OF REPORT: Annual Report

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
October 2014	Annual	13 Sept 2013 – 12 Sept 2014

4. TITLE AND SUBTITLE

Microenvironment-Programmed Metastatic Prostate Cancer Stem Cells (mPCSCs)

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-13-1-0352

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Dean G. Tang

E-Mail: dtang@mdanderson.org

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

The University of Texas MD Anderson Cancer Center

Houston, Texas 78957

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

The main goal of this DOD IDEA project is to further elucidate how orthotopic microenvironment (in the prostate) regulates prostate cancer metastasis. In particular, we wish to understand *the cells-of-origin for PCa metastases*. Clinically, some PCa patients harbor indolent tumors that rarely or only slowly progress whereas other patients have aggressive primary tumors that quickly progress and disseminate. To this end, we implanted multiple GFP or RFP tagged PCa cells either ectopically in the subcutis (s.c) or orthotopically in the dorsal prostate (DP) or anterior prostate (AP) of NOD/SCID mice and followed, in great details, how tumors regenerate and metastasize.

In the past year, we have generated exhaustive data to convincingly demonstrate that the DP- or AP-implanted human PCa cells metastasize much more widely and extensively than s.c-implanted counterparts. We then uncovered the DP and s.c prostate tumors and performed comparative genome-wide transcriptome analysis, which revealed an upregulation of ~600 genes in the DP (i.e., metastasis-prone) tumors that fall into distinct, very informative functional classes. One class of genes, about 50 (~8% of total), is well-known stem cell/CSC regulators and/or markers. Preliminary functional studies on some of the genes implicate their importance in regulating PCa metastasis and in conferring properties of metastatic PCSCs (mPCSCs).

In the coming year, we plan to perform extensive metastasis assays, in several PCa models, to investigate the involvement of representative genes (5-10 genes) in each functional category (including CSC markers; Specific Aim 2) in PCa cell metastasis. Importantly, we'll also examine the metastasis to other organs in addition to the lung and use this functional data to develop an integrative network of "metastasis gene functions". We shall take this 'Metastasis gene signature' to most updated Oncomine database and TCGA to correlate it with the actual patient PCa progression and metastasis. Finally, we plan to prepare a manuscript that summarizes the accumulated data.

15. SUBJECT TERMS

Prostate cancer; metastasis; microenvironment; stem cells; cancer stem cells; orthotopic implantation; ectopic implantation; metastatic prostate cancer stem cells

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	14	19b. TELEPHONE NUMBER *(include area code)*

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

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Department of Defense PCRP IDEA Award
Progress Report (Sept. 13, 2013 to Sept. 12, 2014)

W81XWH-13-1-0352, "Microenvironment-Programmed Metastatic Prostate Cancer Stem Cells (mPCSCs)"

PI: Dean Tang

1. INTRODUCTION:
The subject of our research is to elucidate the cellular and molecular mechanisms underlying prostate cancer (PCa) metastasis. The purpose is to test the overarching hypothesis that *prostatic microenvironment facilitates PCa metastasis by promoting the phenotypic as well as functional manifestations of metastatic prostate cancer stem cells (mPCSCs)*. The scope of the research is:

1) *To perform further functional studies on the genes upregulated in the DP human prostate tumors;*
2) *To test the hypothesis that the DP human PCa cells overexpressing CSC markers possess mPCSC properties; and*
3) *To test the hypothesis that HOXB9 represents a 'master' regulator of mPCSCs and PCa metastasis.*

2. KEYWORDS:
Prostate cancer; metastasis; microenvironment; stem cells; cancer stem cells; orthotopic implantation; ectopic implantation; metastatic prostate cancer stem cells

3. ACCOMPLISHMENTS:

Major Goals of the Project for the first year: 1) To comprehensively demonstrate that the orthotopically implanted human PCa cells manifest more metastatic potential; 2) to uncover the differential gene expression patterns unique to orthotopically implanted metastasis-prone tumors; and 3) to validate and functionally validate some of the representative mPCSC genes

What was accomplished under these goals:

A. *Human PCa cells implanted subcutaneously in NOD/SCID mice manifest significantly higher tumorigenicity than those implanted orthotopically in the dorsal prostate (DP)*.

To systematically understand how human PCa cells regenerate tumors and subsequently spread, we took 3 commonly used cell lines (PC3, Du145, and LNCaP) and 2 xenograft-derived cells (LAPC9 and LAPC4) and performed exhaustive limiting-dilution (tumor regeneration) assays (LDA) by injecting 10 – $1x10^6$ cells either subcutaneously (s.c; ectopic site) or orthotopically in the dorsal prostate (DP). As shown in Tables 1 and 2 and Figure 1a, in all 5 models, human PCa cells injected s.c regenerated more tumors than in the DP.

Table 1. Tumor-initiating frequency (TIF) of PCa cells implanted subcutaneously (s.c) versus in the dorsal prostate (DP) in NOD/SCID mice: A comprehensive study

Cell type#	Cell dose							TIF (95% interval)*	*P* value*
	10^6	$5x10^5$	10^5	10^4	10^3	10^2	10		(fold difference)
Du145									
s.c			4/4	5/6	4/6	3/5		**1/1,570** (1/599-1/4,113)	2.72e-273
DP	3/3 (2x10^6)	3/5	1/4	0/4	0/4			**1/479,971** (1/192,820-1/1,194,4754)	x306
PC3									
s.c				8/8	5/8	1/8	3/15	**1/640** (1/298-1/1,375)	1.49e-06
DP		2/2	3/3	2/4	0/3			**1/15,715** (1/4,212-1/58,637)	x25
LNCaP									
s.c			2/4	1/4				**1/106,500** (1/31,751-1/357,225)	
s.c (with TP)	2/2		3/5	2/4	0/4			**1/69,826** (1/25,573-1/190,661)	x1.5
DP 0/2 (2.5x10^6)	0/4		0/4						
LAPC4									
s.c				6/6	5/8	10/12		**1/340** (1/161-1/716)	7.3e-319
DP		4/5	1/4	0/4	0/4	0/4		**1/329,656** (1/129,567-1/838,741)	x970
LAPC9									
s.c					6/6	3/6		**1/143** (1/48-1/429)	1.14e-291
DP			6/9	4/8	0/9	0/3		**1/58,748** (1/28,327-1/121,841)	x411

#Du145 and PC3 cells were cultured cells injected in 25% Matrigel in NOD/SCID mice. LNCaP cells were cultured cells injected in 25% Matrigel in male NOD/SCID mice with or without testosterone pellet (TP). LAPC4 and LAPC9 cells were acutely purified from xenograft tumors and injected in 25% Matrigel (1:1) in male NOD/SCID mice. All tumor incidences refer to the actual tumors developed/number of injections when animals were terminated in 2-3 months after implantations.

*Tumor-initiating frequency and statistical differences (P values) were determined using the Limdil function of the Satmod package (http:bioinfo.wehi.edu.au/software/elda/index.html)

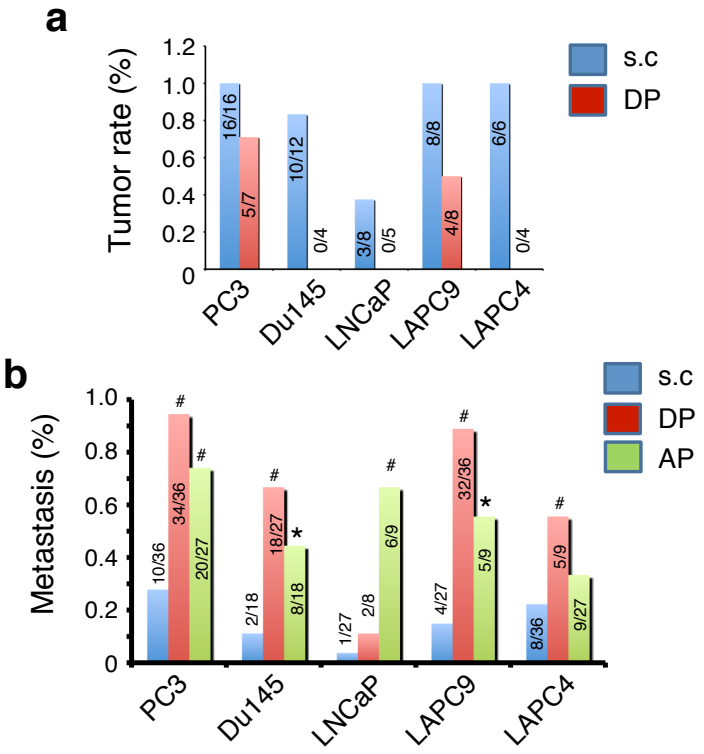


Figure 1. DP-implanted PCa cells have lower tumor-regenerating but higher metastatic potential.
*a. PCa cells indicated (10,000 each) were implanted s.c or in the DP of male NOD/SCID mice. Shown are the tumor-regenerating rates (i.e., total number tumors observed/total number implantations) at the end of the experiments when all animals were terminated (2-3 months). b. Prostate-implanted PCa cells show high metastatic capacity. 100,000 GFP-labeled PCa cells indicated were implanted into the DP or anterior prostate (AP) or s.c. When the animals were terminated, a total of 9 organs (i.e., tibia, femur, lung, pancreas, spleen, kidney, liver, brain, and LN [including draining LN and other LN such as adrenal LN] were removed from each mouse and examined for macroscopic and microscopic metastases. The results were presented as metastatic ratios, i.e., total number organs that showed metastasis/total number organs examined. For example, for PC3 cells, a total of 36 organs were harvested from 4 mice (4 x 9 = 36) that had been implanted with PC3 cells s.c or in the DP. The results showed that 10 of the 36 organs from mice bearing s.c tumors showed metastasis whereas 34 of 36 organs from mice bearing DP tumors showed metastasis. In contrast, 20 of the 27 organs from 3 mice bearing AP-implanted PC3 tumors had metastasis. In the case of LNCaP, only 8 organs from 1 mouse were examined for metastasis. #, $P < 0.001$; and *, $P < 0.05$ compared to corresponding s.c tumors (χ^2 test).*

For example, the tumor-initiating frequency (TIF) for s.c implanted Du145 cells is 1/1,570 (i.e., in every 1,570 Du145 cells injected 1 cell is tumorigenic). In contrast, the TIF for DP-injected Du145 cells is 1/479,971 (Table 1). The difference is highly statistically significant (P = 2.72e-273) with s.c. injected cells being >300 fold more tumorigenic than the DP-injected cells (Table 1). Similar stark differences were noted also in PC3, LAPC4, and LAPC9 models (Table 1). It should be noted that LAPC4 and LAPC9 cells used in these experiments were not cultured cells but purified from the xenograft tumors. LNCaP cells were much less tumorigenic than the other 4 models (Table 1). Taken together, these tumor regeneration assays indicate that *the subcutaneously implanted human PCa cells manifest high tumor-regenerating activity*.

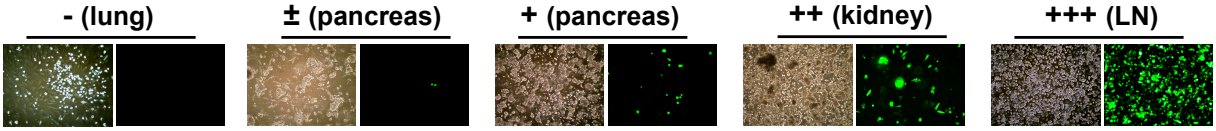
B. *HOWEVER, human PCa cells implanted in the DP manifest much higher metastatic potential than when implanted s.c*.

Subsequently, we made GFP- or RFP-tagged PCa cells for all 5 models, and utilized them to study metastasis. Studying human PCa cell metastasis is very challenging as most metastases in the end organs of immunodeficient mice are micrometastases, which may evade detection by necropsy and regular histologic (HE) analysis. We first carried out 'positive' control experiments by injecting PC3-GFP cells into the tail vein or the heart followed by determining levels of metastasis using several different approaches. *FIRST*, when the animals became moribund, we harvested multiple organs (including lung, kidney, liver, pancreas, brain, spleen,

Table 2. DP-implanted PCa cells show more metastasis than s.c-injected PCa cells

Cell type (Route & number)[a]	Termination[b] (Inj. & term. dates; tumor weights)	Metastasis[c]								
		Femur	Tibia	Lung	Kidney	Liver	Pancreas	Brain	Spleen	LN[d]
PC3-GFP (s.c)										
1,000,000 cells	(Inj. 1/27/06)									
417	term: 02/28/06 (0.2g,0.3g)	-	-	+	-	-	-	-	-	-
419	term: 03/06/06 (0.7g,1.4g)	-	-	+++	+	-	±	-	-	+
414	term: 03/21/06 (3.2g)	-	-	++	+	-	-	-	-	++
418	term: 03/21/06 (1.3g)	-	-	++	-	±	-	-	-	-
PC3-GFP (DP)										
1,000,000 cells	(Inj. 12/27/06)									
431	term: 01/31/07 (0.5g)	+	+	+++	+	±	+	-	+	++
433	term: 01/31/07 (0.3g)	±	±	+++	+	+	+++	±	+	+
428	term: 02/09/07 (1.1g)	-	-	+++	+++	++	+++	±	+++	+++
429	term: 02/09/07 (0.9g)	-	-	+++	++	+	+++	±	++	+++
Du145-GFP (s.c)										
	(Inj: 12/30/05)									
2,000,000 cells	term: 04/18/06 (0.7g)	-	-	±	-	-	-	-	-	-
4,000,000 cells	term: 03/29/06 (4.2g)	-	-	-	-	-	±	-	-	-
Du145-GFP (DP)										
2,000,000 cells	3/3 (Inj: 8/11/05)									
#1	term: 09/29/05	N.D	N.D	+++	++	+	+++	-	++	+++
#2	term: 10/20/05	-	+	+++	++	+	+++	-	++	-
#3	term: 10/29/05	+	-	++	++	+	+++	-	+	-

[a]The indicated numbers of PCa cells were implanted in 50% Matrigel either s.c or into the DP of NOD/SCID mice. Numbers (e.g., 417) beneath refer to animal tags.
[b]Animals were terminated when they became moribund or tumor burden became overwhelming. Both injection (Inj.) and termination (term.) dates are indicated. Note that for s.c injections each animal received either one or two implantations (tumor weights in grams indicated in parentheses).
[c]Metastasis to different organs was evaluated independently by two investigators via observing and enumerating GFP$^+$ cells of the freshly disaggregated whole organs under a fluorescence microscope. Signs of "-" to "+++" refer to relative levels of metastasis in a low-power field. -, no GFP$^+$ cells; ±, <10 GFP$^+$ cells; +, dozens of GFP$^+$ cells; ++, hundreds of GFP$^+$ cells; +++, thousands or tens of thousands of GFP$^+$ cells (see images illustrated below; left, phase; right, GFP). N.D, not determined.
[d]Lymph nodes examined, including renal, caudal, sciatic, mesenteric, inguinal, and pyloric LN.



femur, tibia, and lymph nodes) and screened for and counted GFP-positive (GFP^+) foci on a whole-mount epifluorescence microscope (Liu et al., 2011; *Nature Med* 17(2):211-5). *SECOND*, we disaggregated the whole end organs into single-cell suspension and examined for the existence and semi-quantified the relative abundance of GFP^+ PCa cells under an inverted fluorescence microscope, which allowed the detection of even a single GFP^+ cell. *THIRD*, to further confirm that the GFP^+ cells were actually live cells, we cultured the whole organ-derived cells in PCa cell medium, i.e., RPMI1640 plus 7% FBS for various periods of time. Using this combined approach, we found, as expected, that PC3-GFP cells (1,000,000) injected into the tail veins metastasized only into the lung (not shown). By contrast, intracardiac (IC) injection of 100,000 or 1,000,000 PC3-GFP cells resulted in metastasis to most organs examined, in particular, lung, LN, pancreas, and kidney (not shown). Importantly, both IC-injected PC-GFP and Du145-GFP cells could be cultured up and propagated into live cell colonies (Figure 2).

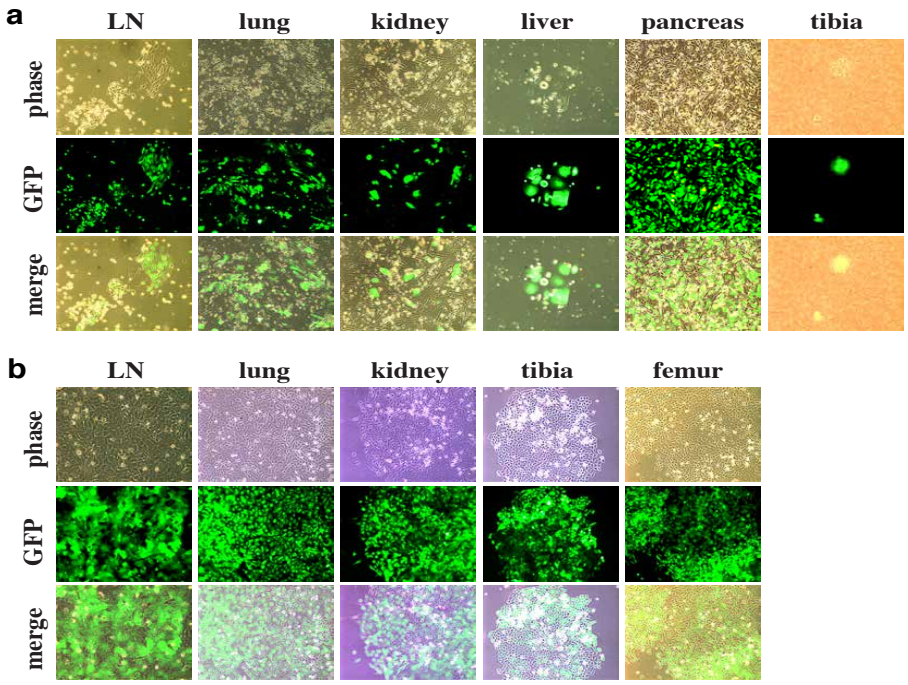


Figure 2. Intracardiacally injected PCa cells recovered from end organs can be cultured up to form colonies.
Shown are representative images of PC3-GFP (a) and Du145-GFP (b) cells cultured (2-3 weeks after plating) from the organs indicated. Animals were injected with 100,000 GFP tagged PCa cells and terminated ~4 months post injection. Organs were disaggregated into single cells, which were plated and cultured in RPMI1640 medium containing 7% FBS. Shown are images of cell colonies cultured for 1-3 weeks. The GFP- cells on the background in some images are the host cells. Original magnifications, x200).

Subsequently, we implanted fluorescently tagged PCa cells s.c or in the DP, and tracked both tumor development and metastasis. As shown in Figure 1b and Table 2, the DP-implanted PCa cells exhibited more prevalent and more extensive metastasis to all organs examined. We also injected human PCa cells in the anterior prostate (AP), an anatomical site that also 'encourages' PCa cell metastasis, and found that our PCa models also exhibited widespread metastasis at the AP site. Interestingly, LNCaP cells injected in the AP appeared to display higher metastatic potential than those injected either s.c or in the DP (Figure 1b). These results, together with many other pieces of data we have accumulated, provide solid evidence that *the orthotopic (i.e., mouse prostate) microenvironment promotes the manifestation of the metastatic potential of human PCa cells*.

C. *Prostate-implanted PCa cells overexpress metastasis, stem cell, and inflammation genes*.
To elucidate the potential mechanisms underlying this interesting 'paradoxical' phenomenon, i.e., human PCa cells in the mouse prostate, despite generating less tumors, manifest much higher metastatic propensity, we harvested 3 pairs of DP and s.c PC3-GFP tumors, respectively, developed in the same mice (to minimize the variations in host factors) and carried out Agilent cDNA microarray expression profiling studies. As illustrated in Table 3, a total of 593 genes representing 423 known and 170 unknown genes were significantly

upregulated. The majority of these upregulated genes, i.e., ~16%, can be functionally classified to the category of 'metastasis-associated', which include genes involved in invasion, motility, angiogenesis, proteolysis, etc (Table 3). Examples include SPP1 (also called osteopontin), which was the most highly upregulated gene, MMPs, IGFBP5, HGF, etc (Table 3).

Table 3. Representative genes upregulated in the DP- vs. s.c-implanted PC3 tumors.

Gene name	Systematic name	Description	Fold increase*	P value[#]
Proteases/invasion/angiogenesis				
SPP1	NM000582	secreted phosphoprotein 1 (osteopontin)	9.4-11.0 (10x)	0.02-0.03
IGFBP5	NM000599	insulin-like growth factor binding protein 5	2.2-4.9 (3x)	0.03-0.04
HGF	NM001010931	hepatocyte growth factor (scatter factor)	2.1-4.5 (10x)	0.04-0.007
SPARC	NM003138	secreted protein, acidic, cysteine-rich (osteonectin)	2.2	$1.2x10^{-6}$
MMP9	NM004994	matrix metallopeptidase 9 (gelatinase B, 92kDa gelatinase, 92kDa type IV collagenase)	5.5-5.9 (10x)	all ~0.03
MMP10	NM002425	matrix metalloproteinase 10 (stromelysin 2)	4.8	0.001
MMP1	NM002421	matrix metallopeptidase 1 (interstitial collagenase)	2.13-2.18 (10x)	all ~0.001
ADAMTS6	NM197941	ADAM metallopeptidase with TSP type 1 motif, 6	4.6	0.004
ADAM23	NM003812	ADAM metallopeptidase domain 23	3.7	0.004
ADAM22	NM021721	ADAM metallopeptidase domain 22	2.3, 2.7	$<10^{-5}$
ESM1	NM007636	endothelial cell-specific molecule 1	6.3, 11.3	both $<10^{-5}$
AGTR1	NM031850	angiotensin II receptor, type 1	4.2	$4.4x1^{-6}$
AGT	NM000029	angiotensinogen (serpin peptidase inhibitor, clade A)	2.0-2.4 (9x)	<0.0001
HIF3A	AK024095	weakly similar to HIF-1α	2.1	$4x10^{-6}$
EDNRA	NM001957	endothelin receptor type A	2.0	0.006
Inflammation/cytokines				
IL-1β	NM000576	interleukin-1β	4.36-4.4 (10x)	all ~0.01
IL1R1	NM000877	interleukin 1 receptor, type I	2.6	0.005
IL13RA2	NM000640	interleukin 13 receptor, alpha 2	2.7	0.005
CXCL7	NM002704	pro-platelet basic protein (PPBP)	5.0	0.005
CXCR4	NM001008540	chemokine (C-X-C motif) receptor 4	4.7-5.0 (10x)	<0.0005
CXCL5	NM002994	chemokine (C-X-C motif) ligand 5	2.2, 3.5	0.04
CCR1	NM001295	chemokine (C-C motif) receptor 1	2.7	0.002
CCR3	NM001837	chemokine (C-C motif) receptor 1	2.1	0.002
CXCL12	NM199168	chemokine (C-X-C motif) ligand 12 (SDF-1)	2.0	0.0003
LCP1	NM002298	lymphocyte cytosolic protein 1 (L-plastin)	4.1	0.01
PLD5	NM152666	phospholipase D family, member 5	3.2	0.0003
TNFS15	NM005118	tumor necrosis factor (ligand) superfamily, member 15	3.0	0.01
ALOX5AP	NM001629	arachidonate 5-lipoxygenase-activating protein	3.0	0.004
NOS2A	NM000625	nitric oxide synthase 2A (inducible)	2.7-3.8 (10x)	0.01-0.009
PLCG2	NM002661	phospholipase C, gamma 2	2.7	0.015
TLR6	NM006068	toll-like receptor 6	2.1	0.0003
C3AR1	NM004054	complement component 3a receptor 1	2.5	$7.6x10^{-5}$
CF1	NM000204	complement factor I	2.5	0.003
CFP	NM002621	complement factor properdin	2.1	0.0004
RGC32	NM014059	response gene to complement 32	2.0,2.1	0.002
C1QTNF2	NM031908	C1q and tumor necrosis factor related protein 2	2.0	0.002
Developmental pathways/stem cell-related				
PROM1	NM006017	prominin 1 (CD133); a stem cell marker	3.3	0.007
FGF7	NM002009	fibroblast growth factor 7 (keratinocyte growth factor)	3.3	$3.7x10^{-7}$
ABCG2	NM004827	ATP-binding cassette, sub-family G	2.7	0.01
CD24	L33930	a CSC marker; related to PCa metastasis	2.5	0.045
KITLG	NM000899	KIT ligand	2.5	0.0008
HOXD13	NM000523	homeobox D13	2.5	0.008
ID3	NM002167	inhibitor of DNA binding 3, dominant negative HLH protein	2.3	0.001
NKX3.1	NM006167	NK3 transcription factor related, locus 1	2.3	$2.4x10^{-5}$
WNT4	NM030761	wingless-type family, member 4	2.2,2.3	0.01, 0.02
FOXJ1	NM001454	forkhead box J1	2.0	0.017
HOXB9	NM024017	homeobox B9	2.0	0.03
TGFBI	NM000358	transforming growth factor, beta-induced, 68kDa	4.2	0.048
BMPR1B	NM001203	bone morphogenetic protein receptor, type IB	3.3	$2.1x10^{-6}$
BAMBI	NM12342	BMP and activin membrane-bound inhibitor	2.4	0.047
INHBA	NM002192	inhibin, beta A (activin A)	2.4	0.001
CHRD	NM003741	chordin	2.4	0.01
TGFB2	NM003238	transforming growth factor, beta 2	2.2	0.04
SMAD1	NM005900	SMAD, mothers against DPP homolog 1	2.1	0.0002
BMP3	NM001201	bone morphogenetic protein 3 (osteogenic)	2.0	0.002

*A total of 745 probe sets representing 593 genes (423 known and 170 unknown) showed ≥2 fold increases. For some genes (e.g., SPP1) multiple probe sets (x) were detected and the range of fold increases was given. The representative genes are listed as functional categories and, among each category, genes are generally listed in descending fold changes according to the functional subclasses, which are color-coded. In each color code, genes in the same subfamily or closely related (in functions) are shaded. See Table S4 for a more complete list of the upregulated genes.

[#]Student's *t*-test.

Strikingly, 8% and 6% of the ~600 upregulated genes can be functionally classified to the categories of 'Development & Stem Cells' & Inflammation & Immune Related' (Table 3). We randomly picked a total of 26 genes from these 3 categories and used qPCR to validate their differential mRNA expression levels in the DP vs. s.c and we were able to validate the higher levels of 24 of the 26 (93%) genes in the DP tumors. We also performed extensive validation studies at the protein levels for many of the upregulated molecules including, among others, SPP1, MMP9, ESM1, and CXCR4 as well as surface molecules such as CD24, CD133, ABCG2, and CXCR4 using several complementary approaches encompassing Western blotting, IHC, ELISA, and FACS (Figure 3).

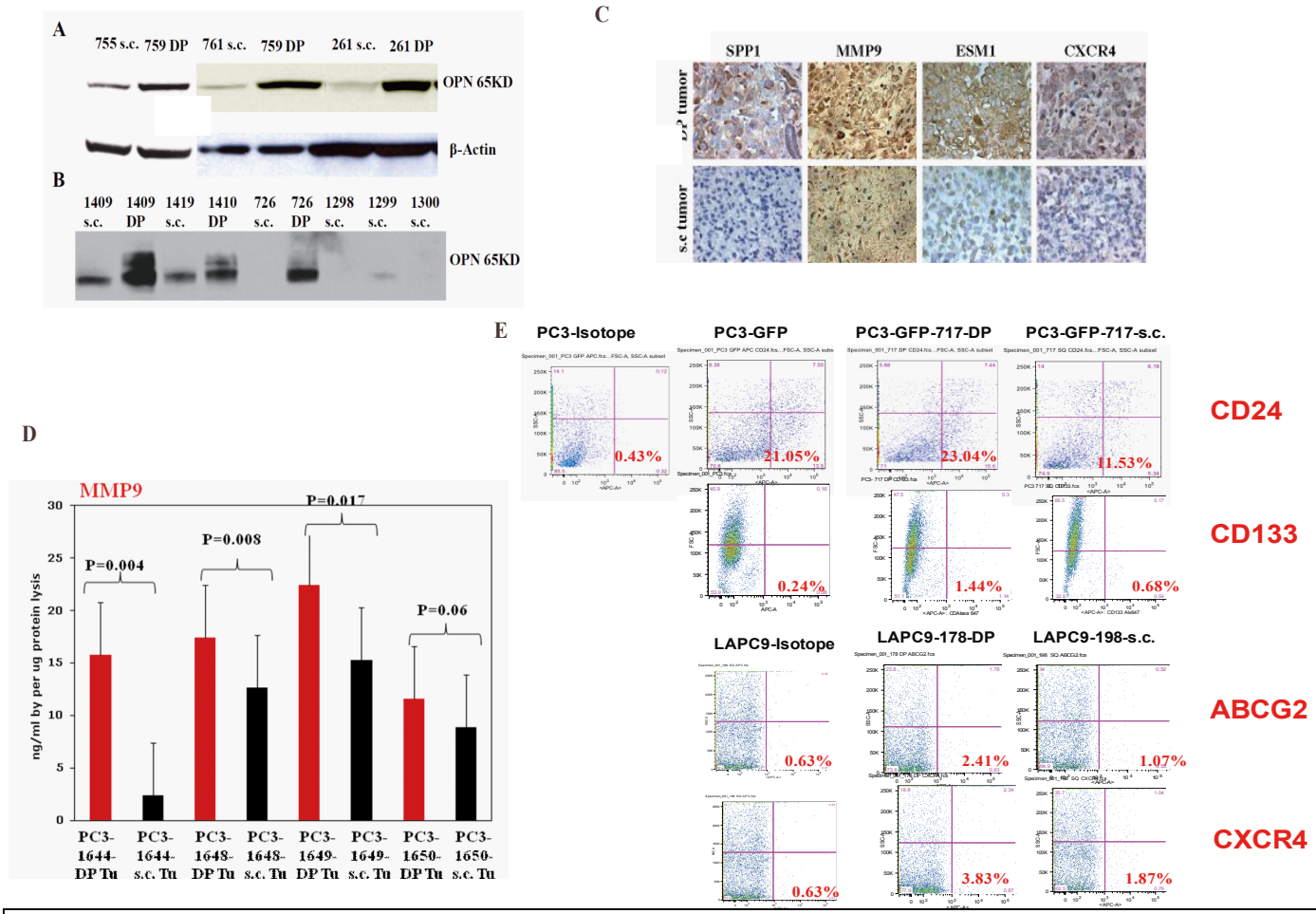


Figure 3. Validation of DP-overexpressed molecules at the protein levels.
(A-B) Overexpression of SPP1 in DP PC3 tumors compared to the s.c PC3 tumors. Indicated are the animal numbers. (C) IHC confirming higher expression levels of SPP1, MMP9, ESM1, and CXCR4 proteins in the DP PC3 tumors (top) compared to the s.c tumors (bottom). (D) Higher levels of MMP9 levels in DP PC3 tumors as evaluated by ELISA assays. (E). Increased surface expression of CD24 and CD133 (prominin-1) in DP PC3 tumors and of ABCG2 and CXCR4 in DP LAPC9 tumors. Shown are the representative flow histograms with the average % marker-positive cells indicated.

To start investigating the potential biological functions of some of the upregulated genes, we employed lentiviral-mediated knockdown and assessed the impact on PCa cell migration/invasions in vitro and lung metastasis in vivo upon transplanting cells in the DP. As shown in Figure 4 (A-B), knocking down MMP9 in LNCaP C4-2 cells inhibited their invasion. Likewise, inhibiting the functions of SPP1 (i.e., osteopontin or OPN) using a neutralizing antibody reduced PC3 cell invasion in Matrigel (Figure 4C-D). Importantly, the MMP9-shRNA significantly inhibited PC3 cells from the prostate to the lung (Figure 4E-F).

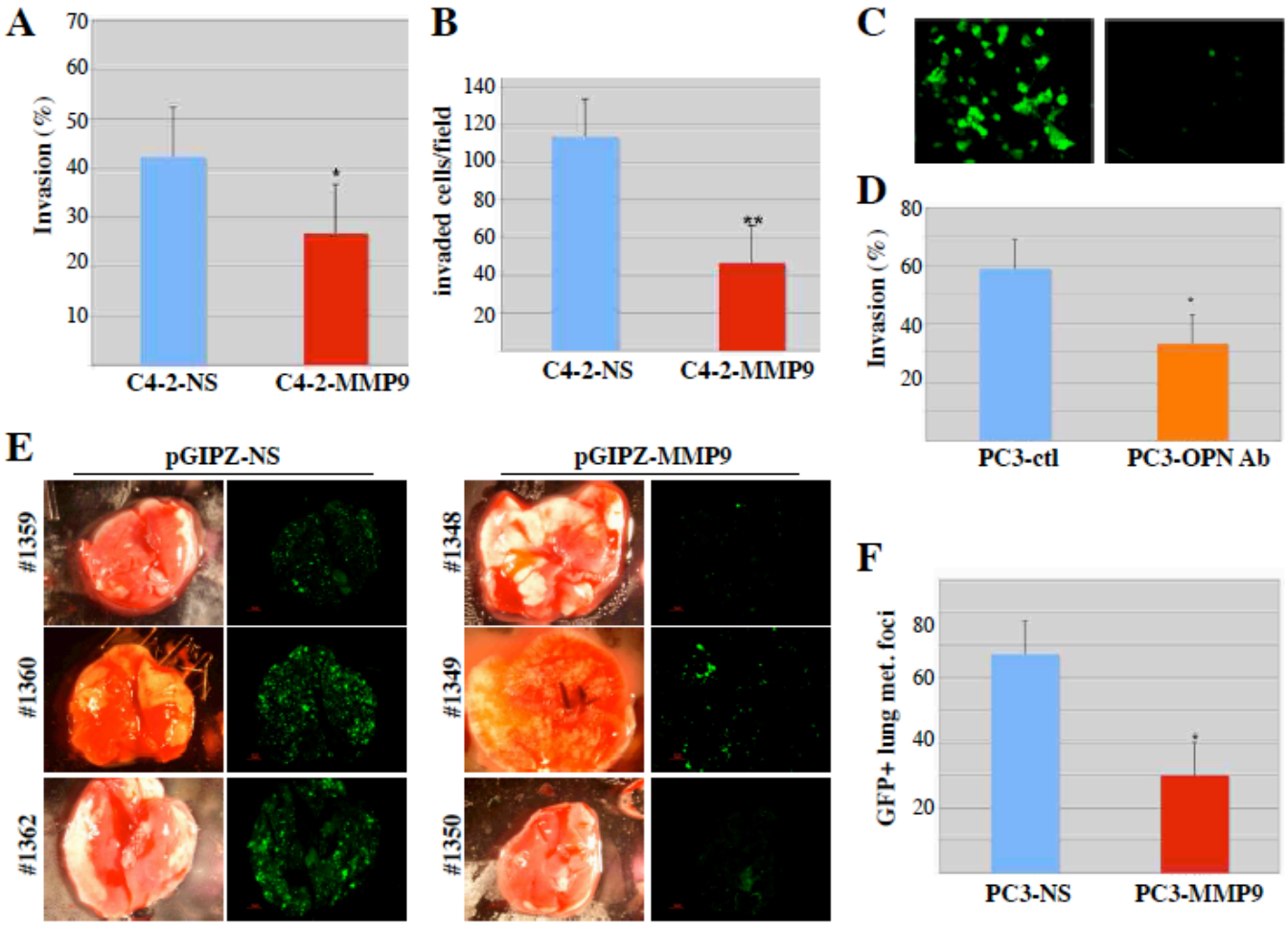


Figure 4. Knocking down MMP9 and SPP1 (OPN) inhibits PC3 cell invasion and metastasis.
(A-B) Knocking down MMP9 inhibits the invasion of LNCaP C4-2 castration-resistant PCa cells, as assessed by Boyden Chamber invasion assays. (C-D) OPN neutralizing antibody (Ab) inhibits PC3 cell invasion. Shown are the representative images (C) and quantification (D). (E-F) MMP9 knockdown inhibits PC3 cell metastasis. PC3 cells were infected with the non-silencing (NS) pGIPZ control vector or the pGIPZ-MMP9 lentivector (both at MOI 1:20). 100,000 cells (48 h after infection) were implanted at the DP (n=6 for each group) and animals were terminated 2 months after injection. Shown are representative images of lung metastasis in 3 animals (E) and quantification (F).

We similarly knocked down several other molecules including OPN, TGFb1, TGFb2, CD44, IL-1b, and ESM1. As summarized in Figure 5, knocking down the expression of OPN, TGFb2, and CD44 in PC3 cells significantly reduced lung metastasis without affecting primary tumor growth. In sharp contrast, in preliminary studies, knocking down TGFb1 and IL-1b did not demonstrate any inhibitory effects (Figure 5B). Knocking down ESM1 partially suppressed lung metastasis although this inhibitory effect did not achieve statistical significance (Figure 5).

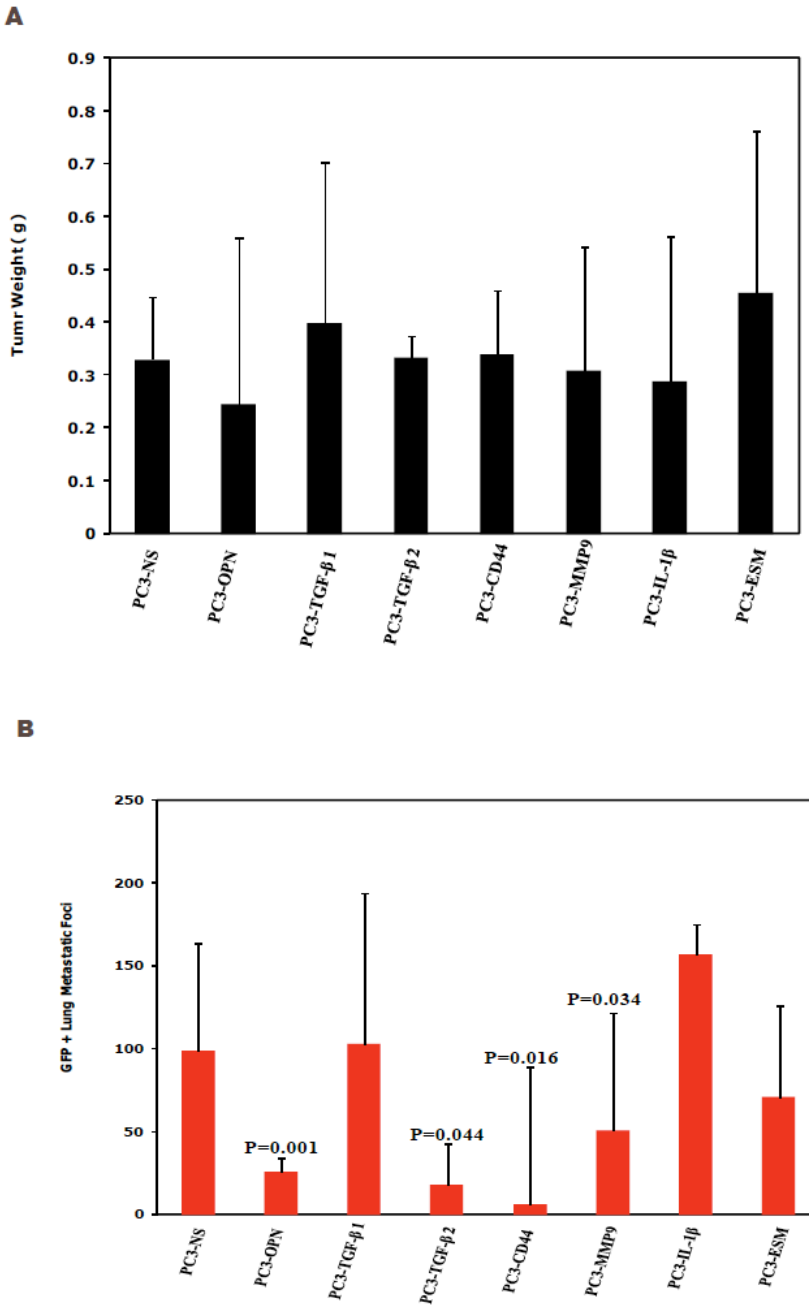


Figure 5. Knocking down some of the upregulated genes inhibits metastasis without inhibiting primary tumor growth in the DP.

Shown are PRELIMINARY RESULTS obtained with knocking down 7 molecules (n=6-9 animals per group for each gene). (A) Tumor weight. (B) Levels of lung metastasis as assessed by counting GFP$^+$ foci (see Liu et al., 2011 for methodology). Bar represent the mean ± S.D. P values are indicated.

What opportunities for training and professional development has the project provided?
Nothing to Report

How were the results disseminated to communities of interest?
Nothing to Report

What do you plan to do during the next reporting period to accomplish the goals?
A. Perform extensive metastasis assays, in several PCa models, to investigate the involvement of representative genes (5-10 genes) in each functional category (including CSC markers; Specific Aim 2) in PCa cell metastasis. Importantly, we'll also examine the metastasis to other organs in addition to the lung.
B. Use this functional data to develop an integrative network of "metastasis gene functions".
C. Take this 'Metastasis gene signature' to most updated Oncomine database and TCGA to correlate it with the actual patient PCa progression and metastasis.
D. Prepare a manuscript that summarizes the accumulated data.
(*It should be noted that as always is the case in science, there could be some minor deviations in our actual flow of work from what was originally proposed in the grant. Nevertheless, the ultimate goal of our research related to this project remains the same, i.e., to determine how primary tumor microenvironment affects the metastatic propensity of PCa cells)*.

4. IMPACT:

a. **What was the impact on the development of the principal discipline(s) of the project?**
For the first time, we have generated convincing data that when human PCa cells are implanted subcutaneously in immunodeficient NOD/SCID mice, they readily regenerate tumors but rarely metastasize. In contrast, orthotopically implanted human Pca cells generate less tumors but extensively metastasize. This message should greatly impact how future studies on modeling human Pca metastasis should be designed and executed.

b. **What was the impact on other disciplines?**
The findings here should also have bearing on similar metastasis studies of other solid tumors.

c. **What was the impact on technology transfer?**
Nothing to Report

d. **What was the impact on society beyond science and technology?**
Nothing to Report

5. CHANGES/PROBLEMS:
Nothing to Report

6. PRODUCTS:
The current project intersects with several other projects in the lab, all of which have a common goal, i.e., to dissect PCa cell heterogeneity and to elucidate the role of different subpopulations of PCa stem/progenitor cells in tumor initiation, maintenance, progression,

drug resistance, and metastasis. The following published manuscripts have cited the partial support of the DOD grant:

Badeaux MA, Jeter CR, Gong S, Liu B, Suraneni MV, Rundhaug J, Fischer SM, Yang T, Kusewitt D, and **Tang DG**. In vivo functional studies of tumor-specific retrogene NanogP8 in transgenic animals. ***Cell Cycle*** 12: 2395-2408, 2013.

Shi Y, Liu C, Liu X, **Tang DG**, and Wang J. The microRNA miR-34a inhibits non-small cell lung cancer (NSCLC) growth and the CD44hi stem-like NSCLC cells. ***PLoS One***, 9(3): e90022, 2014. PMCID: PMC3942411

Liu B, Badeaux MD, Choy G, Chandra D, Shen I, Jeter CR, Rycaj K, Lee CF, Person MD, Lu C, Chen Y, Shen J, Jung SY, Qin J, and **Tang DG**. Nanog1 in NTERA-2 and recombinant NanogP8 from somatic cancer cells adopt multiple protein conformations and migrate at multiple M.W species. ***PLoS One***, 9(3): e90615, 2014. PMCID: PMC3944193

Rycaj K and **Tang DG**. Cancer stem cells and radioresistance. ***Int J Radiat Biol***. 90:615-621, 2014.

Suraneni MV, Moore JR, Zhang D, Badeaux M, Macaluso MD, DiGiovanni J, Kusewitt D, and **Tang DG**. Tumor-suppressive functions of 15-Lipoxygenase-2 and RB1CC1 in prostate cancer. ***Cell Cycle*** 13(11): 1798-1810, 2014.

Jin M, Zhang T, Liu C, Badeaux MA, Liu B, Liu R, Jeter C, Chen X, Vlassov AV, and **Tang DG**. MicroRNA-128 suppresses prostate cancer by inhibiting BMI-1 to inhibit tumor-initiating cells. ***Cancer Res***, 74:4183-4195, 2014.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS:

Name:	Xin Chen
Project Role:	Post doc
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	12
Contribution to Project:	Dr. Chen performed most metastasis related and gene knockdown assays.
Funding Support:	This DOD grant

Name:	Hseuh-Ping (Eva) Chao
Project Role:	Graduate Student
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	4
Contribution to Project:	Eva was involved in bioinformatically analyzing differentially expressed genes
Funding Support:	This DOD grant

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?
Nothing to Report

What other organizations were involved as partners?
Nothing to Report

8. SPECIAL REPORTING REQUIREMENTS:
N/A

9. APPENDICES:
N/A